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# Interleukin 4, Interferon- $\gamma$ , and Prostaglandin E Impact the Osteoclastic Cell-Forming Potential of Murine Bone Marrow Macrophages\*

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## ABSTRACT

Interleukin 4 (IL-4) is an immune cytokine that inhibits bone resorption in mice and suppresses osteoclastic cell formation *in vitro* through an undefined mechanism. In this report, we have established the cellular identity of the IL-4 target cell using a variety of bone marrow/stromal cell coculture methods. Initially, we found that the majority of IL-4's inhibition of osteoclastic cell formation was due to its effect on bone marrow cells, not stromal cells. Consequently, bone marrow macrophages were used as osteoclastic cell progenitors after they had been transiently exposed to IL-4 (48 h), before the addition of stromal cells, 1,25-dihydroxyvitamin D<sub>3</sub>, and dexamethasone. In this circumstance, IL-4 impaired subsequent osteoclastic cell formation, suggesting that the macrophage may be potentially targeted by

many factors known to influence osteoclast formation. Consequently, we discovered that interferon- $\gamma$  (IFN $\gamma$ ), prostaglandin E (PGE), and cell-permeant cAMP analogs also impacted osteoclastic cell formation when used to selectively treat bone marrow macrophages. IFN $\gamma$  suppressed osteoclastic cell formation, whereas PGE and cAMP analog treatment led to the formation of significantly enlarged osteoclastic cells. Importantly, PGE antagonized the inhibitory effects of both IL-4 and IFN $\gamma$  on the osteoclastic cell-forming potential of bone marrow macrophages. Collectively, these findings establish bone marrow macrophages as osteoclastic cell precursors with the degree of their commitment to the osteoclast pathway sensitive to the effects of soluble mediators, including IL-4, IFN $\gamma$ , and PGE. (*Endocrinology* 136: 2367–2376, 1995)

INTERLEUKIN 4 (IL-4) is a 20-kilodalton immunoregulatory glycoprotein that is produced and secreted by activated T lymphocytes and mast cells. This cytokine functions as a growth and/or differentiation factor for a wide variety of cells of hematopoietic lineage (1–7). In addition to these effects, IL-4 has been shown to influence skeletal metabolism (8). In this study, IL-4 inhibited the *in vitro* bone resorption induced by a wide variety of agents [PTH, PTH-related peptide (PTHrP), 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>], IL-1 $\alpha$ , IL-1 $\beta$ , and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)], which in excess have been implicated in both osteoporosis and hypercalcemia of malignancy (9). This inhibitory effect of IL-4 on bone resorption was neutralized by the addition of a monoclonal anti-IL-4 antibody. These observations have been extended to the intact animal, as IL-4 has been shown to antagonize bone resorption stimulated in mice by infusions of PTHrP or by PTHrP- and IL-1 $\alpha$ -expressing tumors (10).

Although these observations established IL-4 as a potent antiosteolytic factor, the mechanism(s) underlying its effects

remained poorly characterized. Because IL-4 inhibited the resorption-inducing effects of such a diverse group of osteolytic substances, we reasoned that this cytokine impacted essential resorptive processes. In support of this hypothesis, we documented that IL-4 significantly antagonizes osteoclast generation *in vitro* (11) in an *in vitro* hematopoietic model of osteoclastic cell formation using bone marrow cell/stromal cell cocultures. These findings were confirmed by others, who showed that IL-4 inhibits osteoclastic cell generation in whole bone marrow and stromal cell/spleen cell cocultures (12, 13).

Although the ability of IL-4 to impair osteoclastic cell generation was a novel observation, the processes and cells impacted by this cytokine that disrupt osteoclastic cell formation remain unclear. Because IL-4 has profound complex effects on the proliferation and maturation of a variety of hematopoietic cells (5, 7) that may be mediated by the effects of lymphokine on stromal cells (6), the principal objective of this study was to determine the IL-4 cellular target within the myriad of cells that comprise our coculture model. Furthermore, we reasoned that the identification of the IL-4 target cell must precede further attempts to characterize the mechanism of IL-4's inhibitory effects on *in vitro* osteoclastic cell formation. Once we had accomplished this task, we directed interferon- $\gamma$  (IFN $\gamma$ ) and PGE, agents with previously described effects on *in vitro* bone resorption and osteoclastic cell formation (13–17), against the putative IL-4-sensitive cell to determine whether these agents also targeted the same cell. Finally, we present an updated model of *in vitro* osteoclast

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formation, originally outlined by Suda *et al.* (18), that incorporates our findings.

## Materials and Methods

### Mice and cell lines

C3H/HEN mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). ST2 cells (stromal cell line) were obtained from RIKEN Cell Bank (Tsukuba, Japan), maintained in  $\alpha$ -Minimum Essential Medium supplemented with 10% heat-inactivated fetal calf serum ( $\alpha$ 10-MEM), passaged weekly after trypsinization, and used up to passage 10.

### Reagents

Recombinant IL-4 was expressed and purified from baculovirus-transfected SF-9 (*Spodoptera frugiperda*) cell culture supernatants as previously described (19, 20) and was stored at  $-80^{\circ}\text{C}$ . The biological potency of IL-4 was determined using the HT-2 (murine T cell line) proliferation assay (21), with the amount of material stimulating half-maximal proliferation equal to 1 U. Macrophage colony-stimulating factor (M-CSF) was purified from L929 cell-conditioned medium by a modification (22) of the method of Stanley and Heard (23). Murine IFN $\gamma$  was obtained from Genzyme (Cambridge, MA). Pronase was purchased from Boehringer Mannheim (Indianapolis, IN).  $1,25\text{-(OH)}_2\text{D}_3$  was kindly provided by Milan Uskokovic (Hoffman LaRoche, Nutley, NJ). Unless otherwise stated, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

### Bone marrow cell preparation

Bone marrow cells were prepared by a modification (11) of a previously described method (22, 24). Note that this method involves an initial overnight incubation to deplete the bone marrow cells of adherent cells, and this step includes the addition of M-CSF (500 U/ml).

### Stromal cell (ST2 cells or calvarial osteoblastic cells)/bone marrow cocultures

Cocultures of bone marrow cells and ST2 cells were performed in several different ways (see Fig. 1), all of which resulted in the formation of osteoclastic cells. In the first method (Fig. 1A), termed the continuous coculture method, ST2 cells ( $1 \times 10^5$  cells/ml) were cultured for 24 h in  $\alpha$ 10-MEM in 96-well (0.2 ml) or 48-well (0.5 ml) plates. At this time, the ST2 culture supernatants were discarded, and the previously prepared nonadherent bone marrow cells ( $1 \times 10^6$  cells/ml) in  $\alpha$ 10-MEM containing  $10^{-8}$  M  $1,25\text{-(OH)}_2\text{D}_3$  and  $10^{-7}$  M dexamethasone were added to the ST2 cell cultures (0.2 ml, 96-well plate; 0.5 ml, 48-well plate). The cultures were maintained for 10 days, with medium changed twice weekly.

In the second method, termed the nonadherent cell method (Fig. 1B), bone marrow and stromal cells were cultured for 4 days before their combination to allow for a pretreatment period. To prevent their adherence during this time, nonadherent bone marrow cells were cultured in Teflon beakers in  $\alpha$ 10-MEM supplemented with 1000 U/ml M-CSF. ST2 cells were cultured in  $\alpha$ 10-MEM in T-25 flasks. After a 4-day incubation, ST2 cells were trypsinized and suspended in  $\alpha$ 10-MEM. Then, ST2 cells and bone marrow cells were combined and cultured in  $\alpha$ 10-MEM containing  $10^{-8}$  M  $1,25\text{-(OH)}_2\text{D}_3$  and  $10^{-7}$  M dexamethasone for 10 additional days.

In the third method, termed the adherent cell method (Fig. 1C), the nonadherent bone marrow cells, isolated after overnight incubation in M-CSF, were grown on tissue culture plates in the presence of 1000 U/ml M-CSF. After 3 additional days, nonadherent cells were rinsed away, and the remaining adherent cells (termed day 4 cells) were exposed to various conditions in the presence of M-CSF (1000 U/ml) for 48 h. After these treatments, adherent cells were again rinsed, and ST2 cells were added ( $1 \times 10^5$  cells/ml; 0.5 ml; 48-well plate well) in  $10^{-8}$  M  $1,25\text{-(OH)}_2\text{D}_3$  and  $10^{-7}$  M dexamethasone containing  $\alpha$ 10-MEM. The cultures were maintained for an additional 8 days, with medium changed once.

### Bone resorption assay (pit assay)

Thin disc-shaped wafers ( $7 \times 1$  mm) of cortical bone were prepared from transverse slices bovine femurs (diaphysis) with a low speed diamond saw (Buehler, Evanston, IL) and stored in absolute ethanol at  $4^{\circ}\text{C}$ . Before their use, the slices were washed with sterile PBS and placed in 48-well plate wells. Day 1 nonadherent bone marrow cells ( $5 \times 10^5$  cells/well) were cultured on the bone slices in 0.5 ml  $\alpha$ 10-MEM containing 1000 U/ml M-CSF for 3 days (protocol similar to those in Fig. 1C). Nonadherent cells were then rinsed from the wells, and various treatments were added. After 2 additional days, these treatments were rinsed away, and ST2 cells ( $5 \times 10^4$  cells/well),  $10^{-8}$  M  $1,25\text{-(OH)}_2\text{D}_3$ , and  $10^{-7}$  M dexamethasone were added. After an additional 8 days (medium changed every 4 days), the bone pits (resorption lacunae) were visualized using a variation of a previously described method (25). The slices were exposed to 0.1 M sodium hydroxide for 15 min. The disc surfaces were vigorously rinsed with this solution, using a pipette to remove the cell layer from the bone. The slices were then rinsed in 1% (wt/vol) sodium borate and stained with toluidine blue solution [1% (wt/vol) in the borate solution] for 10 min. The slices were briefly rinsed in dye-free borate solution, air-dried, and then mounted on glass slides with Acrytol mounting medium (Surgipath Medical Industries, Richmond, IL). Pits appeared as darkly staining, clearly margined areas using light microscopy.

### Enzyme chemistry: tartrate-resistant acid phosphatase (TRAP) staining

The presence of generated osteoclastic cells was evaluated by cytochemical detection of TRAP using a commercial kit. A cell that was TRAP

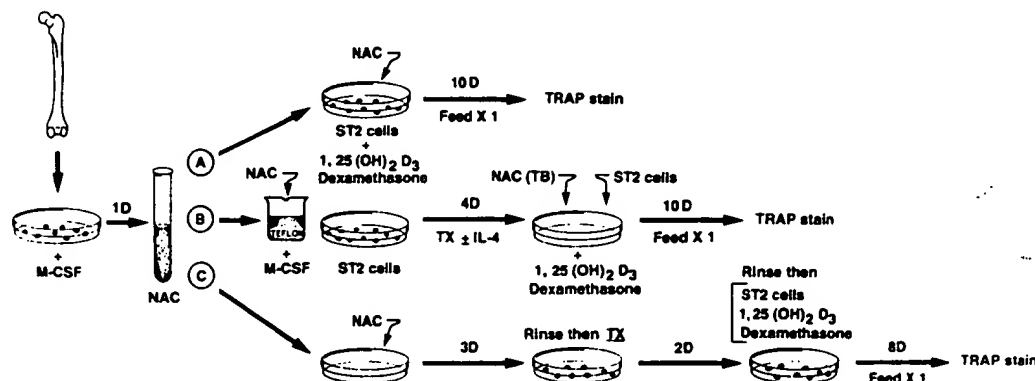


FIG. 1. Cell culture methods used for osteoclastic cell development. A, Continuous coculture method. B, Nonadherent cell method. C, Adherent cell method. D, Day; NAC, nonadherent cells; TB, Teflon beaker.

positive and contained three or more nuclei (TRAP-positive MNC) was considered an osteoclastic cell.

### Image analysis

Osteoclastic cells on plastic dishes and toluidine blue-stained bone pits were viewed with a Nikon Diaphot (Nikon Corp., Melville, NY) or Labophot microscope (Nikon), respectively, equipped with a Sony XC77 camera (Sony Corp., Tokyo, Japan). The camera was connected to a digitizing scientific frame grabbing card (model LG-3, Scion Corp., Frederick, MD) located in a Macintosh II computer. The osteoclastic cell and pit images were traced with the assistance of a digital tablet (ArtZ, WACOM, Vancouver, WA) and image analysis software (NIH IMAGE, version 1.47, public domain). To obtain measurements in units of measure, the system was calibrated using a slide with an inscribed line of known length.

### [<sup>125</sup>I]IL-4 binding assay and IL-4 receptor Northern blots

[<sup>125</sup>I]IL-4 binding assays and Northern blots screened for the presence of IL-4 receptor transcripts were done as previously described (26).

### Statistics

In certain experiments, the data were statistically assessed using analysis of variance. The differences between groups were tested for significance using Fisher's protected least significant difference test, with the cut-off level set at 5%. The Kolmogorov-Smirnov test was used for the pit area data due to the apparent skewness of the data. These analyses were performed with the assistance of a computer program (Statview 4.0, Abacus Concepts, Berkeley, CA).

## Results

In an earlier account (11), we demonstrated that IL-4 inhibited osteoclastic cell formation using the continuous bone marrow cell/ST2 cell coculture system (Fig. 1A) over a cytokine range that also stimulated T cell proliferation. To determine whether this IL-4 effect was time of addition dependent, IL-4 was added at the time of culture initiation or on days 4 and 7 thereafter, and osteoclastic cell number was determined on day 10. As shown in Fig. 2, IL-4 inhibited osteoclastic cell formation only when it was added at the initiation of coculture (day 1). The addition of IL-4, when delayed to either day 4 or 7 of coculture, failed to impair osteoclastic cell formation. These results suggested that the principal effects of IL-4 occurred during the first 4 days of the coculture period through its effects on one of the many cells present in this complex culture system.

To further delineate the cell population impacted by IL-4, we designed a cross-over experiment (see Fig. 1B), the aim of which was to determine whether the bone marrow cells or ST2 cells were the IL-4 cellular targets. In these experiments, nonadherent bone marrow cells were cultured for 4 days in medium containing 1000 U/ml M-CSF with and without IL-4 (50 U/ml) in Teflon beakers, to which macrophages poorly adhere. In parallel with these bone marrow cultures, ST2 cells were cultured in medium with and without IL-4 (50 U/ml) for 4 days. The ST2 and bone marrow cells were then combined and cultured for 10 days in the presence or absence of IL-4 (50 U/ml). As summarized in Table 1, continuous IL-4 treatment (IL-4 in bone marrow cell, stromal cell, and their subsequent cocultures) led to a 70% inhibition of osteoclastic cell number compared to the control value. Importantly,

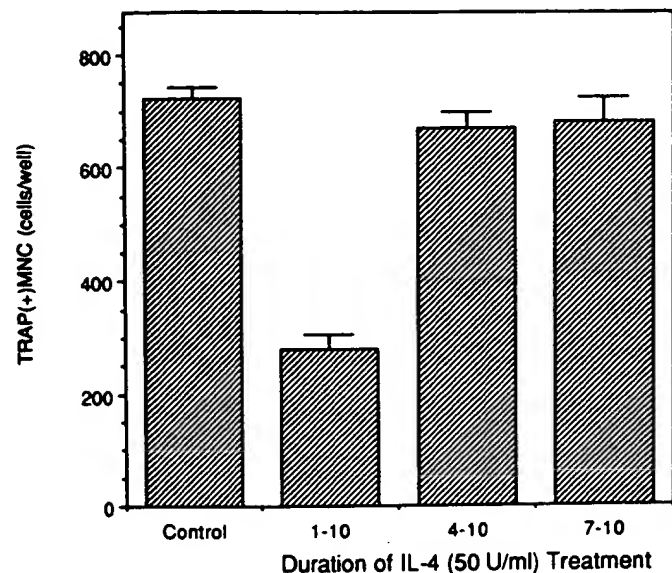


Fig. 2. IL-4's inhibitory effect on osteoclastic cell formation requires early exposure. ST2/nonadherent bone marrow cell cultures were established as described (Fig. 1A), and IL-4 (50 U/ml) was added at culture initiation or on days 4 and 7 thereafter. Osteoclastic cells were enumerated as described (see *Materials and Methods*). The results represent the mean ( $n = 4$ )  $\pm$  SD number of osteoclastic cells per 96-well plate well. The 1-10 group is statistically different from all others ( $P < 0.0001$ ).

TABLE 1. Effects of IL-4 on osteoclastic cell formation is largely mediated by cells within the bone marrow population

First culture <sup>a</sup>		Coculture <sup>b</sup>	TRAP-positive MNC (cells/well) <sup>c</sup>	% Inhibition <sup>d</sup>
BM cells	ST2 cells			
— <sup>e</sup>	—	—	693.2 $\pm$ 52.9	
—	—	+	504.0 $\pm$ 27.2	27.3
+	—	—	318.3 $\pm$ 34.7	54.1
+	—	+	249.5 $\pm$ 35.6	64.0
—	+	—	691.0 $\pm$ 23.8	0.3
—	+	+	483.3 $\pm$ 34.3	30.3
+	+	—	346.3 $\pm$ 53.4	50.1
+	+	+	210.3 $\pm$ 22.0	69.7

<sup>a</sup> Nonadherent bone marrow (BM cells) and ST2 cells were cultured separately for 4 days, as described in *Materials and Methods*.

<sup>b</sup> The BM cells ( $5 \times 10^5$  cells) and ST2 cells ( $5 \times 10^4$  cells) were then combined and cultured for 10 days in the presence of  $10^{-8}$  M 1,25-(OH) $_2$ D $_3$  and  $10^{-7}$  M dexamethasone.

<sup>c</sup> Osteoclastic cells were identified as TRAP-positive cells containing three or more nuclei. The results are reported as the mean ( $n = 4$ )  $\pm$  SD.

<sup>d</sup> This value is derived as follows:  $(693.2 - x)/693.2 \times 100$ . Three-way analysis of variance established that IL-4 treatment of BM cells ( $P < 0.0001$ ) or the presence of IL-4 in cocultures ( $P < 0.0001$ ) resulted in significant differences. IL-4 treatment of ST2 cells alone did not lead to significant effects.

<sup>e</sup> The + and — indicate that the culture was treated with either IL-4 (50 U/ml) or medium alone, respectively.

most of this inhibition (~77%) could be explained by the effect of IL-4 on bone marrow cells, because IL-4 bone marrow cell pretreatment alone led to a 54% reduction of osteoclastic cell number. Pretreatment of stromal cells with IL-4 essentially failed to impair osteoclastic cell number. These results suggested that the impact of IL-4 on a cell within the bone marrow cell compartment resulted in reduced oste-

oclastic cell formation in this system. The possibility that IL-4's apparent selective action on bone marrow cells was due to the lack of IL-4 receptor expression by ST2 or osteoclastic cell-containing cultures was excluded by experiments demonstrating that both of these cultures contained IL-4 receptor messenger RNA (mRNA; Northern blots) and cell surface IL-4 receptors ( $[^{125}\text{I}]\text{IL-4}$  binding; data not shown).

The objective of the next series of experiments was to further define the IL-4 cellular target in the bone marrow cell population. Based on the observations that 1) tissue macrophages functioned as osteoclastic cell precursors (27); 2) M-CSF-deficient animals failed to produce osteoclasts (28, 29); and 3) we inhibited osteoclastic cell formation by selectively treating bone marrow cells raised with M-CSF and IL-4 (Table 1), we reasoned that the IL-4 cellular target was a M-CSF-responsive cell in the marrow compartment. To test whether bone marrow macrophages *per se* could function as osteoclastic cell progenitors, we cultivated the day 1 nonadherent bone marrow cells in M-CSF-containing medium for 3 days and then rinsed away the nonadherent cells. This results in the isolation of a M-CSF-dependent adherent cell population consisting principally of macrophages (30). When ST2 cells,  $1,25\text{-(OH)}_2\text{D}_3$  (10 nM), and dexamethasone (100 nM) were added, multinucleated TRAP-positive cells with features identical to those generated in the previous experiments developed over 8 additional days (data not shown). These findings indicated that bone marrow macrophages possessed the capacity to differentiate into osteoclastic cells given the appropriate conditions and, therefore, may be selectively targeted by agents known to alter *in vitro* osteoclastic cell formation, such as IL-4.

To test this hypothesis, day 4 bone marrow macrophages were exposed to various concentrations of IL-4 after the nonadherent cells had been rinsed from the wells. After an additional 48 h, the IL-4 treatment medium was removed, and ST2 cells were added together with  $1,25\text{-(OH)}_2\text{D}_3$  (10 nM) and dexamethasone (100 nM). Osteoclastic cell number was determined 8 days later. As shown in Fig. 3, IL-4 dose dependently inhibited osteoclastic cell development, with maximal inhibition (~75%) at 200 U/ml IL-4 and the 50% inhibitory dose ( $\text{ID}_{50}$ ) of IL-4 being approximately 22 U/ml. The IL-4  $\text{ID}_{50}$  in this circumstance was quite similar to the value we observed in experiments in which we had tested IL-4 in the continuous coculture model (IL-4  $\text{ID}_{50}$  = 10 U/ml) (11).

These findings suggested that bone marrow macrophages were targeted by IL-4 and that this lymphokine altered (in this case impaired) the cells' osteoclastic cell-forming potential. To determine whether the osteoclastic cell-forming potential of the bone marrow macrophage at this stage of its *in vitro* differentiation was selectively impacted by IL-4, we tested other agents that had been previously shown to either inhibit (IFN $\gamma$ ) (13) or stimulate (PGE $_1$ , PGE $_2$ , and cAMP) (31) osteoclastic cell formation *in vitro*. In contrast to the previous osteoclastic cell development models that used unfractionated whole bone marrow cultures or stromal cell/spleen cell cocultures where these factors have been examined, we directed these agents directly against bone marrow macrophages transiently (48 h) before the addition of stromal cells,

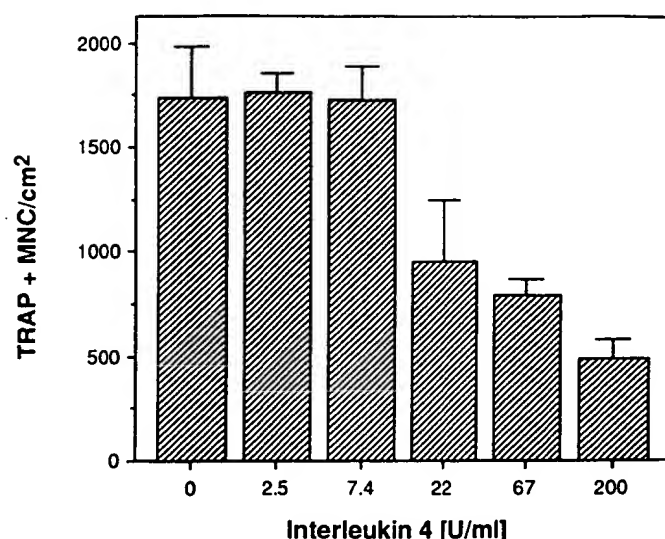


FIG. 3. IL-4 inhibits the osteoclastic cell-forming potential of bone marrow macrophages. Day 4 bone marrow macrophages raised in M-CSF-containing medium were exposed to various concentrations of IL-4 for 48 h before the addition of ST2 cells,  $1,25\text{-(OH)}_2\text{D}_3$ , and dexamethasone as described (adherent cell method, Fig. 1C). Osteoclastic cells were counted as described and are presented as the mean  $\pm$  SEM. Compared to the control, the 22 ( $P < 0.0061$ ), 67 ( $P < 0.0016$ ), and 200 ( $P < 0.0001$ ) U/ml treatment groups were statistically significant.

$1,25\text{-(OH)}_2\text{D}_3$  (10 nM), and dexamethasone (100 nM). As shown in Fig. 4, IFN $\gamma$  inhibited osteoclastic cell formation dose dependently, with an  $\text{ID}_{50}$  between 10–100 U/ml. Maximum inhibition (~85%) occurred with 1000 U/ml IFN $\gamma$ .

PGE $_1$ , on the other hand, had different effects on osteoclastic cell formation (Fig. 5) when used to treat bone marrow macrophages. Prostanoid exposure actually re-

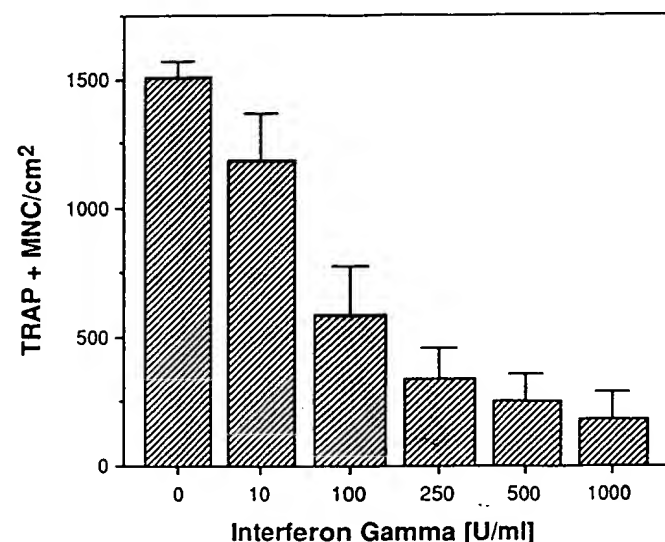


FIG. 4. IFN $\gamma$  inhibits the osteoclastic cell-forming potential of bone marrow macrophages. Bone marrow macrophages were prepared and treated as described in Fig. 3, except that various amounts of IFN $\gamma$  were employed instead of IL-4. Osteoclastic cells were counted as described and are presented as the mean  $\pm$  SEM. All IFN $\gamma$  treatments at 10 U/ml and above led to significant declines in osteoclastic cell number (10 U/ml,  $P < 0.0101$ ; 100–1000 U/ml,  $P < 0.0001$ ).

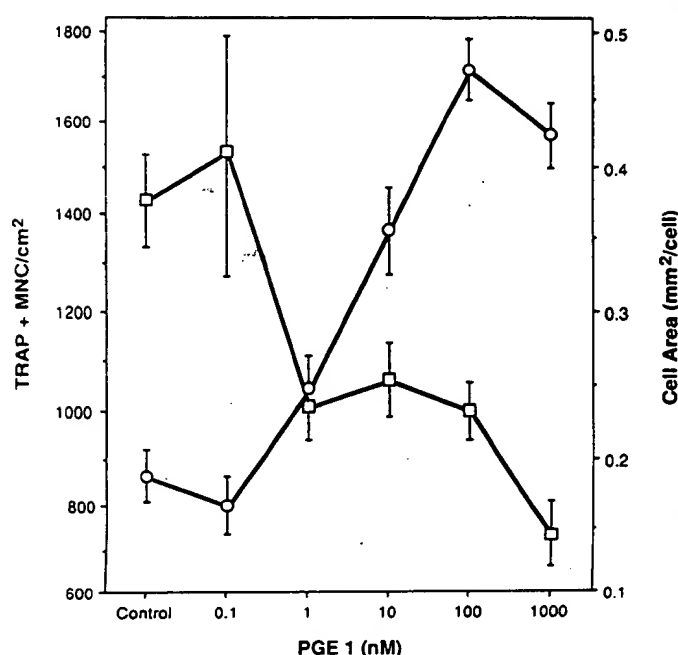


FIG. 5. PGE<sub>1</sub> impacts the osteoclastic cell-forming potential of bone marrow macrophages. Bone marrow macrophages were prepared and treated as described in Fig. 3, except that various amounts of PGE<sub>1</sub> were employed instead of IL-4. Osteoclastic cells were counted (□), and the areas (○) of the largest osteoclastic cells ( $n = 40/\text{treatment}$ ) in each treatment group were measured as described and are presented as the mean  $\pm$  SEM. Cell area was significantly different from the control value at all PGE<sub>1</sub> levels above 1 nM PGE<sub>1</sub> (1 nM,  $P < 0.0745$ ; 10, 100, and 1000 nM,  $P < 0.0001$ ).

duced osteoclastic cell number in a concentration-dependent manner, with an ED<sub>50</sub> of about 1 nM. The greatest effect was seen at 1  $\mu\text{M}$  PGE<sub>1</sub>, where osteoclastic cell number was 50% of the control value. In contrast to its dampening effect on osteoclastic cell number, however, PGE had a marked impact on the area of the individual osteoclastic cells that developed from the bone marrow macrophages, as the PGE<sub>1</sub>-treated cultures contained numerous huge osteoclastic cells. In fact, these large osteoclastic cells became nearly confluent on the plastic surface beneath the stromal cell layer (see Fig. 6). To make a more quantitative assessment of this effect, we used an image analysis method to measure the cell area of the largest osteoclastic cells (40 largest/treatment group) formed in the wells subsequent to PGE<sub>1</sub> exposure. These results are also shown in Fig. 5. As the graph demonstrates, there is an inverse relationship between cell number and size. That is, as the osteoclastic cell number decreased in response to higher PGE<sub>1</sub> levels, the osteoclastic cells that developed became larger in size, with the cross-over point of these parameters being about 1 nM. At 100 nM PGE<sub>1</sub>, the cells that developed were 2–3 times larger in area than those in control wells. In accordance with the increased cell area, the larger cells that formed subsequent to PGE<sub>1</sub> exposure contained more nuclei than the controls (data not shown), indicating that an increased degree of cell fusion had occurred. Essentially the same results were obtained for PGE<sub>2</sub> (data not shown).

Akatsu *et al.* (31) previously showed that the effects of PGE on osteoclastic cell formation in a whole bone marrow culture



FIG. 6. PGE<sub>1</sub> treatment leads to osteoclastic cells having an increased cell area. Bone marrow macrophages were prepared and treated as described in Fig. 1C with and without 1  $\mu\text{M}$  PGE<sub>1</sub> during the 48-h intermediate period. The cultures were TRAP stained 8 days later, and photomicrographs ( $\times 20$  magnification) were prepared. A, Control; B, PGE<sub>1</sub>. The calibration bar represents 250  $\mu\text{m}$ .

model of osteoclastic cell formation could be approximated by use of the cell-permeant cAMP analog, (Bu)<sub>2</sub>cAMP (31). To determine whether cAMP could play a role in the adherent cell model we developed, we exposed day 4 adherent bone marrow macrophages to various concentrations of either 8-bromo- (8-Br-cAMP) or *N*(6)-benzoyl-cAMP for 48 h, as we had done with the PGEs. After rinsing, ST2 cells, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and dexamethasone were added as outlined (Fig. 1C). Consistent with these previous results, both cAMP analogs (Fig. 7, A and B) had an impact on osteoclastic cell formation, with their effects being qualitatively similar to those observed with PGE<sub>1</sub>. At high levels of the cAMP analogs (1 mM), osteoclastic cell number was decreased ( $\sim 50\%$ ), yet cell area was markedly increased (2- to 3-fold), just as had occurred when PGE<sub>1</sub> was employed. The cAMP effect diminished as the concentrations of these agents decreased. At less than 10  $\mu\text{M}$ , the effects of cAMP on osteoclastic cell number and size disappeared.

To exclude the possibility that the cell size-enhancing effect of PGE and cAMP was just an *in vitro* artefact without functional significance, we examined the impact of these



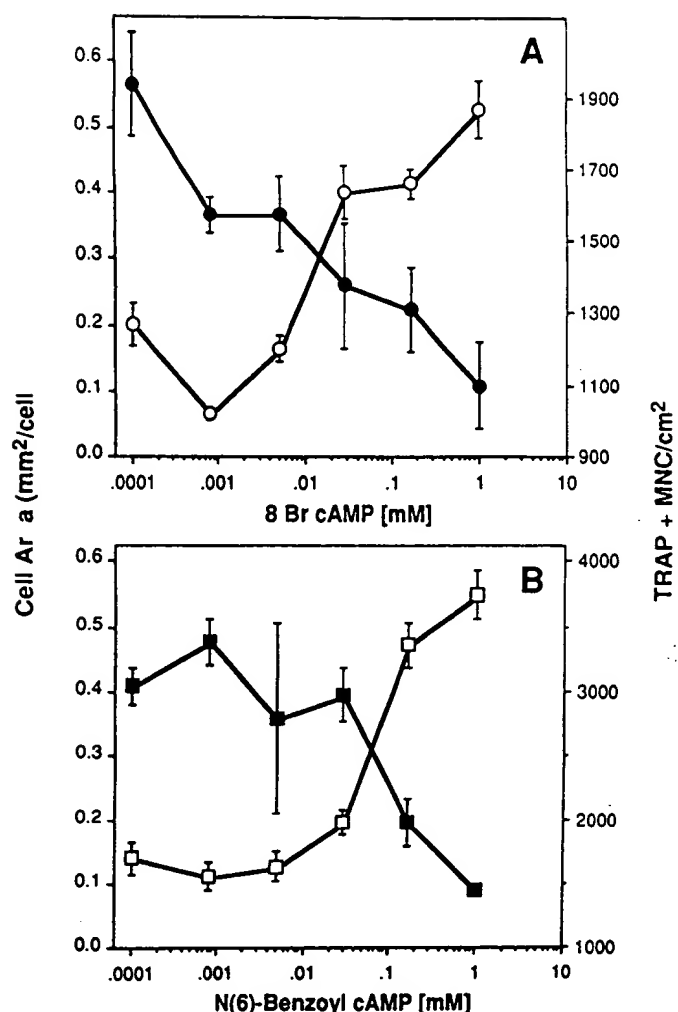


FIG. 7. cAMP derivatives impact the osteoclastic cell-forming potential of bone marrow macrophages. Bone marrow macrophages were prepared and treated as described in Fig. 3, except that various amounts of either 8-Br-cAMP (A) or N(6)-benzoyl-cAMP (B) were added instead of IL-4. Osteoclastic cells were counted (● and ■), and the areas (○ and □) of the largest osteoclastic cells ( $n = 30$ /treatment) in each treatment group were measured as described and are presented as the mean  $\pm$  SEM. For cell area for the 8-Br-cAMP group, the treated cells were significantly larger than controls in the 1 mM ( $P < 0.0001$ ), 0.17 mM ( $P < 0.0001$ ), and 0.028 mM ( $P < 0.0001$ ) treatment groups. For cell area for the N(6)-benzoyl-cAMP group, the treated cells were significantly larger than controls in the 1 mM ( $P < 0.0001$ ) and 0.167 mM ( $P < 0.0001$ ) treatment groups.

treatments on resorption lacunae formation. For this experiment, we used the adherent cell method (Fig. 1C), and instead of culturing the cells on plastic, we cultured the bone marrow cells on slices of bovine cortical bone. On day 4, we rinsed nonadherent cells from the wells, and for 2 days the cultures were treated with PGE<sub>1</sub> and PGE<sub>2</sub> (1  $\mu$ M), 8-Br-cAMP (1 mM), or medium alone. All conditions contained M-CSF (1000 U/ml) to maintain macrophage viability. After this intermediate period, ST2 cells, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and dexamethasone were added for an additional 8-day period. The pit areas were then evaluated by image analysis after the bone slices had been appropriately stained. Figure 8 shows the results of this experiment, in which 100 pits from each

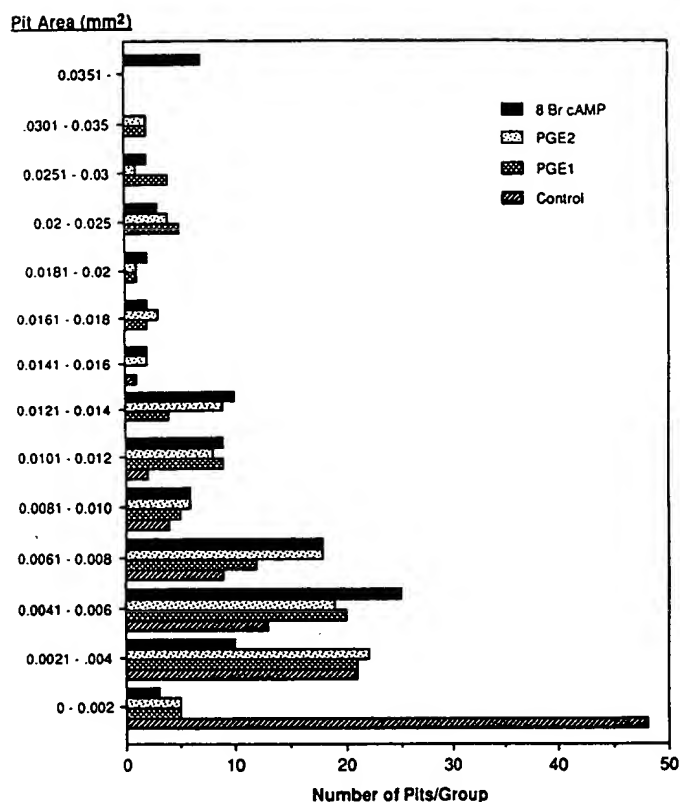


FIG. 8. PGE<sub>1</sub>, PGE<sub>2</sub>, and 8-Br-cAMP impact resorption lacunae area. Bone marrow macrophages were prepared on slices ( $n = 3$ /group) of bovine cortical and treated as described with PGE<sub>1</sub> (1  $\mu$ M), PGE<sub>2</sub> (1  $\mu$ M), 8-Br-cAMP (1 mM), or control medium before the addition of ST2 cells, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and dexamethasone as described (Fig. 1C). After an additional 8 days, the slices were processed and stained to demonstrate resorption lacunae (pits). The area of 100 pits from each group was determined by image analysis and plotted in the histogram. The pits in all treatment groups were significantly larger than the control using the Kolmogorov-Smirnov test ( $P < 0.0001$ ).

treatment condition were measured. Most of the pits that formed under control conditions (~70%) were less than 0.004 mm<sup>2</sup> in area. In contrast, the majority of pits that formed in the PGE (PGE<sub>1</sub>, ~75%; PGE<sub>2</sub>, ~75%) and 8-Br-cAMP (~85%) were larger than 0.004 mm<sup>2</sup>. No pit in the control group exceeded 0.016 mm<sup>2</sup>, whereas pits exceeding this size were found in the groups treated with PGE<sub>1</sub>, PGE<sub>2</sub>, and 8-Br-cAMP. These data suggest that under the conditions examined, increased osteoclastic cell area on plastic and the capacity for the cells to form pits with an increased area on bone are associated.

In contrast to suppressing the osteoclastic cell-forming potential of bone marrow macrophages, both IL-4 and IFN $\gamma$  have been shown to augment the capacity of these cells to perform various immune activities (32–35). Interestingly, PGE and cAMP derivatives have been found to antagonize many of the immune-stimulating effects of both of these lymphokines (36–39). To determine whether PGE<sub>1</sub> and 8-Br-cAMP could also antagonize the inhibitory effect of IL-4 on the osteoclastic cell-forming potential of bone marrow macrophages, we added these agents together to the adherent day 4 bone marrow macrophages before the introduction of conditions leading to terminal osteoclastic cell differentia-

tion. As shown in Fig. 9A, PGE $_1$  partially reversed the inhibitory effects of IL-4 on osteoclastic cell number. Additionally, 8-Br-cAMP also reversed the inhibitory effects of IL-4, similar to what was observed with PGE $_1$ . Importantly, both PGE $_1$  and 8-Br-cAMP augmented osteoclastic cell area about 4- to 5-fold (Fig. 9B) even when combined with IL-4.

To determine whether the inhibitory effects of IFN $\gamma$  on osteoclastic cell formation were sensitive to PGE treatment, day 4 bone marrow macrophages were exposed for 48 h to various IFN $\gamma$  concentrations in the presence and absence of 1  $\mu$ M PGE $_1$ . Eight days after the introduction of ST2 cells, 1,25-(OH) $_2$ D $_3$ , and dexamethasone, osteoclastic cell number and size were evaluated. As shown in Fig. 10A, PGE $_1$  antagonized the inhibitory effects of IFN $\gamma$  on cell number when

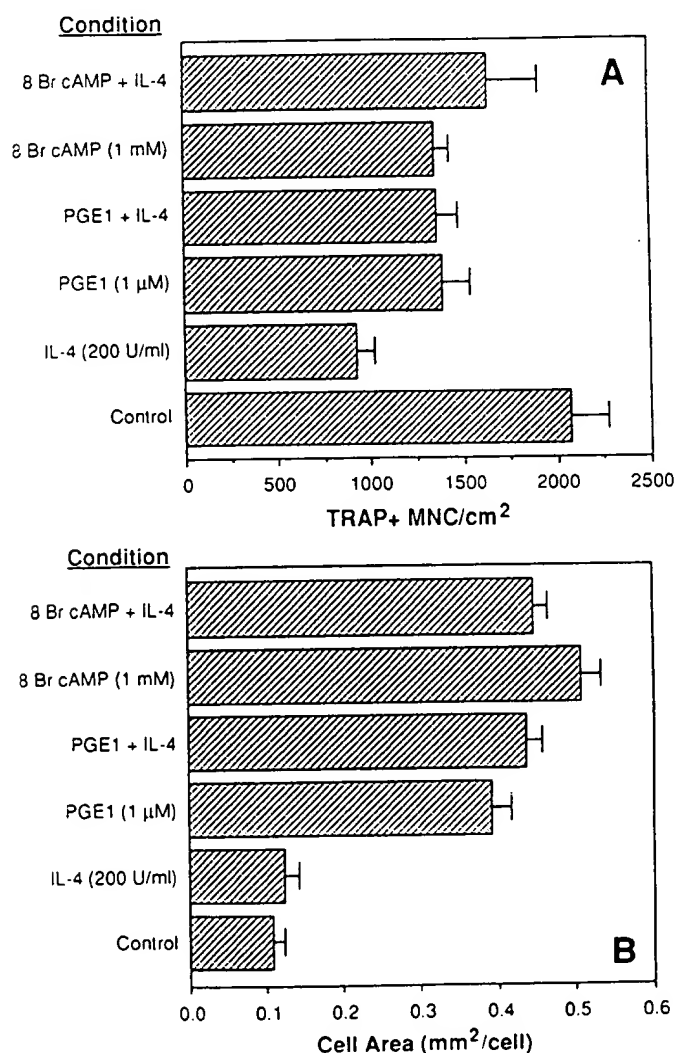


FIG. 9. PGE $_1$  and 8-Br-cAMP antagonize the effects of IL-4 on osteoclastic cell formation. Bone marrow macrophages were treated with control medium or medium with IL-4, PGE $_1$ , 8-Br-cAMP or various combinations thereof for 48 h before the addition of ST2 cells, 1,25-(OH) $_2$ D $_3$ , and dexamethasone, as depicted in Fig. 1C. Osteoclastic cell number (A) and area (B) were assessed as described, and the data are presented as the mean  $\pm$  SEM. For cell area, the *P* values for the groups were as follows: IL-4 plus PGE $_1$  vs. IL-4, *P* < 0.0001; IL-4 plus 8-Br-cAMP vs. IL-4, *P* < 0.0001; PGE $_1$  vs. control, *P* < 0.0001; 8-Br-cAMP vs. control, *P* < 0.0001; IL-4 vs. control, *P* = NS.

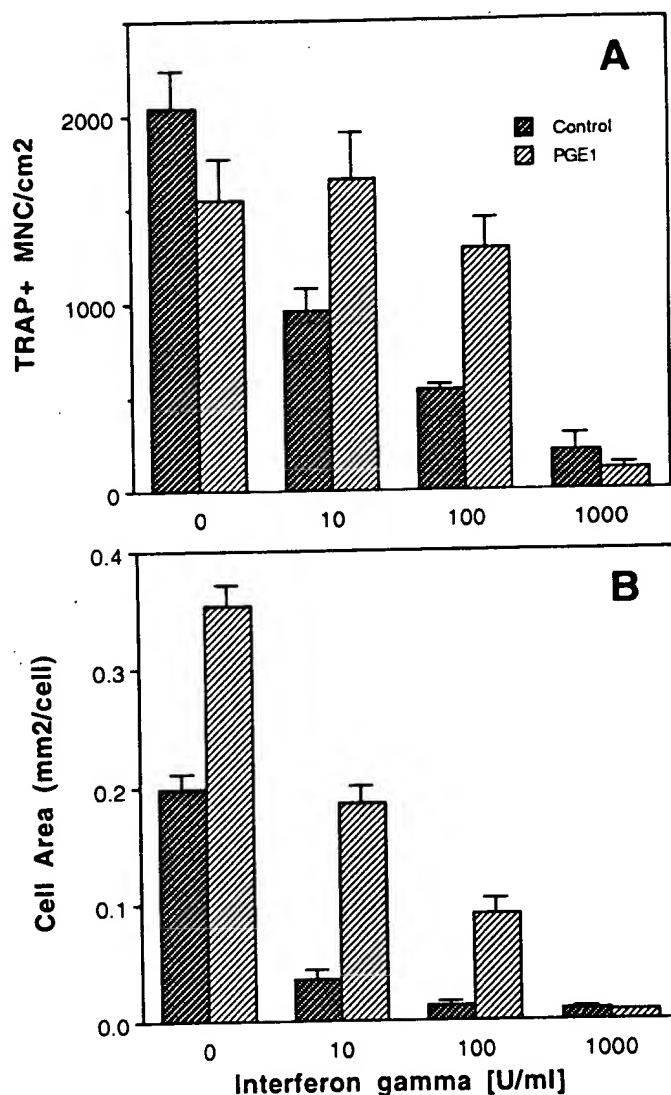


FIG. 10. PGE $_1$  antagonizes the effects of IFN $\gamma$  on osteoclastic cell formation. Bone marrow macrophages were treated with control medium or medium with the indicated amounts of IFN $\gamma$ , PGE $_1$  (1  $\mu$ M), or various combinations thereof for 48 h before the addition of ST2 cells, 1,25-(OH) $_2$ D $_3$ , and dexamethasone, as depicted in Fig. 1C. Osteoclastic cell number (A) and area (B) were assessed as described, and the data are presented as the mean  $\pm$  SEM. For cell area, the *P* values for the groups were as follows: 1000 U/ml IFN $\gamma$  plus PGE $_1$  vs. 1000 U/ml IFN $\gamma$ , *P* = NS; 100 U/ml IFN $\gamma$  plus PGE $_1$  vs. 100 U/ml IFN $\gamma$ , *P* < 0.0001; 10 U/ml IFN $\gamma$  plus PGE $_1$  vs. 10 U/ml IFN $\gamma$ , *P* < 0.0001; PGE $_1$  vs. control, *P* < 0.0001.

this lymphokine was present at 10 and 100 U/ml. PGE $_1$  treatment also led to the development of larger osteoclastic cells in both control and IFN $\gamma$ -treated cultures, as demonstrated in Fig. 10B. In contrast, PGE $_1$  was essentially without effect on cells treated with 1000 U/ml IFN $\gamma$ , which is a massive level of the factor.

### Discussion

In this study, we have shown that the inhibitory effects of IL-4 on osteoclastic cell formation using the continuous coculture model of osteoclastogenesis occurred early in the



culture period. Based on the cross-over experiment, the IL-4 cellular target was present in the bone marrow cells. Further fractionation of the bone marrow cell population, using adherence and M-CSF responsiveness as selection criteria, resulted in a cell population that formed osteoclastic cells after ST2 cell and steroid additions. Using these adherent cells, we observed that IL-4 inhibited osteoclastic cell formation, which is similar to what we had observed when this cytokine was employed in the cross-over experiments or continuous coculture system (11). These data suggest that the IL-4-sensitive target was the bone marrow macrophage based on the cell's adherence and the fact that they were derived from the bone marrow using the monocyte-macrophage lineage-specific growth factor, M-CSF (40). Furthermore, bone marrow cells prepared in this manner have previously been shown to express mannose receptors (22), a phagocytic receptor that is expressed widely in cells of the mononuclear phagocyte system (41).

Surprisingly, treatment of ST2 cells with IL-4 before their combination with bone marrow cells did not lead to impaired osteoclastic cell formation. In hematopoiesis, Peschel *et al.* (6) reported that bone marrow-derived stromal cell monolayers pretreated with IL-4 inhibited hematopoietic colony formation. However, they also demonstrated that glucocorticoids (hydrocortisone) blocked the IL-4-induced inhibitory effect if added during either the IL-4 preincubation phase or the colony formation stage. Because we employed dexamethasone in the coculture medium or added stromal cells after the bone marrow macrophages had already been treated with IL-4 in the adherent cell model, it is unlikely that the production of IL-4-induced inhibitory factors, similar to what Peschel *et al.* (6) observed, could have played a role in our cultures.

Because the monocyte-macrophage presented itself as the likely IL-4 target, we queried whether this cell could be influenced by other agents known to influence *in vitro* osteoclastic cell generation. PGEs and IFN $\gamma$  are factors that have been shown to stimulate (31) and inhibit (13), respectively, osteoclastic cell formation in murine bone marrow cultures. Consistent with these previous reports, we found that both PGE and IFN $\gamma$  impacted osteoclastic cell formation when used selectively on bone marrow macrophages before the introduction of ST2 cells, 1,25-(OH) $_2$ D $_3$ , and dexamethasone. Surprisingly, the PGEs dose dependently reduced the number of osteoclastic cells. However, prostanoid exposure led to the formation of huge osteoclastic cells that contained increased numbers of nuclei. Because increased cell fusion has led to the presence of the larger osteoclastic cells, it is probable that it has indirectly reduced the number of countable osteoclastic cells. Therefore, we still consider PGEs as stimulants of osteoclastic cell formation, with the most pronounced effect in our system resulting in increased osteoclastic cell size. The capacity for PGE to induce this effect *in vitro* is consistent with the results of Holtrop *et al.* (42), who found osteoclasts formed in PGE-treated fetal rat long bones *in vitro* to be of increased size relative to controls.

In agreement with prior reports, cAMP analogs produced effects similar to those of PGE on both generated osteoclastic cell number and size. Furthermore, it appears that increased osteoclastic cell size on plastic may predict increased resorp-

tive activity, because resorption lacunae that formed on bone slices in PGE- and cAMP-treated cultures were larger in area than those that formed in control cultures. To determine whether the enlarged osteoclastic cells resorbed more bone would require three-dimensional studies of the resorption lacunae that would establish the volume of bone removed. In any event, these findings confirm and extend previous reports examining the effects of PGEs on *in vitro* osteoclastic cell formation (17, 31) by 1) identifying a PGE-sensitive osteoclastic cell progenitor within the bone marrow cell compartment and 2) demonstrating that the same cells targeted by PGE respond to cAMP analogs in a similar manner.

Most importantly, however, PGE $_1$  antagonized the osteoclastic cell-inhibiting effects of IL-4 and IFN $\gamma$ . Although these observations suggested that IL-4- and IFN $\gamma$ -mediated suppression of osteoclastic cell formation could have been mediated by impaired PGE production in our cultures, the failure of indomethacin (1 and 10  $\mu$ M) to produce this inhibitory effect (data not shown) when used during the same 48-h treatment period essentially discounts this possibility.

The agents we tested have effects on osteoclastic cell proliferation that appear to be dissociated from their impact on M-CSF-driven bone marrow macrophage proliferation. For instance, IFN $\gamma$  (43) and PGE (44) are both known to inhibit macrophage proliferation, yet these agents have opposing effects on the osteoclastic cell-forming potential of the same cells. Along these same lines, the fact that IL-4 can enhance macrophage proliferation in the presence of M-CSF is also known (45), and we confirmed this observation (data not shown). Therefore, we conclude that the effects of IL-4, PGE, IFN $\gamma$ , and the cAMP analogs on osteoclastic cell formation are not a direct reflection of their impact on M-CSF-driven bone marrow macrophage proliferation.

Despite the fact that our studies have led to the identification of macrophages as the target cell in our system, the mechanism(s) driven by IL-4, IFN $\gamma$ , and PGE remains undefined. A provocative hypothesis that ties together many of our observations can be proposed based on a recent report describing the osteopetrosis that develops in *c-fos* knockout mice (46). In this study, the researchers present convincing evidence that osteoclast differentiation from macrophages is blocked in *c-fos*-deficient animals, indicating that *c-fos* induction in osteoclast precursors may be pivotal for terminal differentiation. That this block may occur at the level of the macrophage is supported by the presence of numerous macrophages in the bone marrow of the *c-fos*-deficient osteopetrotic animals. These results raise the obvious possibility that alterations in macrophage *c-fos* induction could explain the findings observed in our study. In support of this contention, both IL-4 and IFN $\gamma$  have been shown to suppress *c-fos* induction in monocyte/macrophages (47, 48), and in our system, both agents inhibit osteoclastic cell formation from bone marrow macrophages. Furthermore, agents that elevate or mimic cAMP in macrophages, such as PGE and 8-Br-cAMP, have been shown to cause prolonged elevations of macrophage *c-fos* mRNA levels (48, 49), and in our model they stimulate osteoclastic cell development. These data raise the possibility that inflammatory cytokines and lipids may influence osteoclast development in part through their ef-

fects on signaling pathways that alter *c-fos* expression in macrophages.

Based on our results, we formulated a variation of a model (see Fig. 11) of *in vitro* osteoclastic cell formation originally presented by Suda *et al.* (18). In our adapted model, the monocyte-macrophage is a candidate osteoclastic cell progenitor subject to local conditions, including the presence of immune and inflammatory mediators. In constructing this model, we took into consideration previous studies of lymphokine-mediated macrophage activation. Previous investigators have established that both IFN $\gamma$  and IL-4 have immunomodulatory effects on macrophages, including increases in both class II antigen expression (32) and, in the case of IL-4, induction of multinucleation (12, 50). Furthermore, PGEs and cAMP analogs antagonize the effects of both lymphokines on macrophages (38, 51) while, based on our experiments, priming the same cells to differentiate into osteoclastic cells, provided that local conditions are met. Therefore, we postulate that the relative actions of the lymphokines and PGs on the macrophage determine the cell's ultimate capacity to differentiate into an osteoclastic cell. Interestingly, the effects of these agents appear to occur during an intermediate period of differentiation before the introduction of 1,25-(OH) $_2$ D $_3$ , dexamethasone, and stromal cells. These latter factors and cells apparently need to be present during a proposed terminal differentiation period that concludes with the appearance of osteoclastic cells.

With regard to further studies, the adherent cell method provides a model to test other potential osteoclastogenesis-influencing agents in a defined (albeit artefactual) manner. There are four different identifiable periods in this method that could be targeted, as shown in Figs. 1 and 11, including early (days 0–1), growth (days 2–4), intermediate differen-

tiation (days 5–6), and terminal differentiation (days 7–14) periods. Our initial results with IL-4 focussed our attention at the intermediate differentiation period, where we proceeded to test other osteoclastogenesis-modifying factors. Although our findings identify a narrow window of time (days 5 and 6, intermediate differentiation period) as a factor-sensitive interval, we did not examine the possibility that the same agents explored above could have effects if used during any of the other intervals.

In the case of IL-4, Riancho *et al.* (12) showed that IL-4's inhibition of osteoclastic cell formation was mediated by the cytokine's effects on their culture before the appearance of osteoclastic cells. They could only theorize that IL-4 acted on putative uncommitted osteoclastic cell precursors in the bone marrow or spleen because they had employed mixed cell populations in their cultures. In contrast to speculation, we identified the IL-4 cellular target in the marrow compartment and showed that the same cell is responsive to other treatments.

In summary, our results identify the bone marrow macrophage as a mononuclear phagocyte with the potential to function as an efficient osteoclastic cell precursor. The degree to which the cell is actually committed to the osteoclast pathway appears to be influenced by soluble mediators produced by cells of the immune system. Because IL-4 and IFN $\gamma$  appear to direct macrophage differentiation toward immune activities and away from osteoclastic cell formation, there may be a common specific step in the mechanisms driven by these factors, sensitive to PGEs and potentially other cAMP-generating conditions, that is a pivotal determinant of macrophage differentiation. The elucidation of this putative maturation "checkpoint" could potentially provide the basis for antiresorptive therapies.

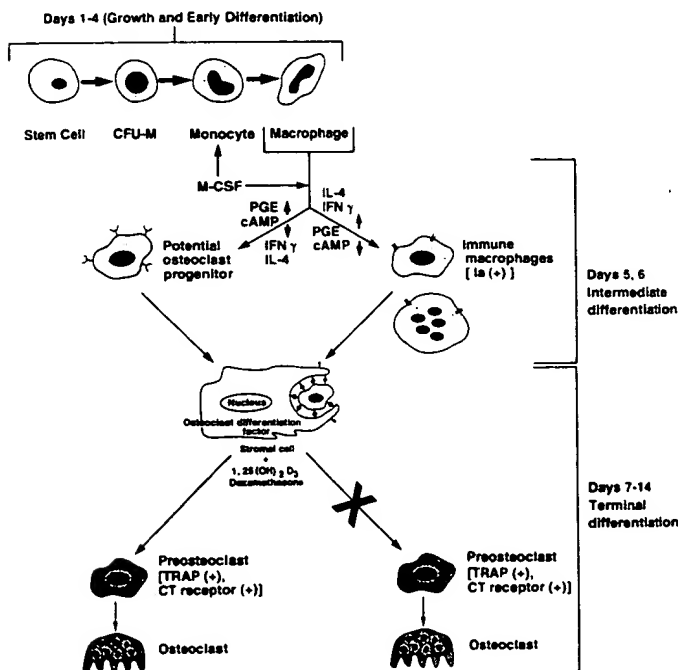


FIG. 11. Model of osteoclastic cell formation from bone marrow macrophages [adapted from Suda *et al.* (18)]. Ia, Class II antigen; CT, calcitonin.

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